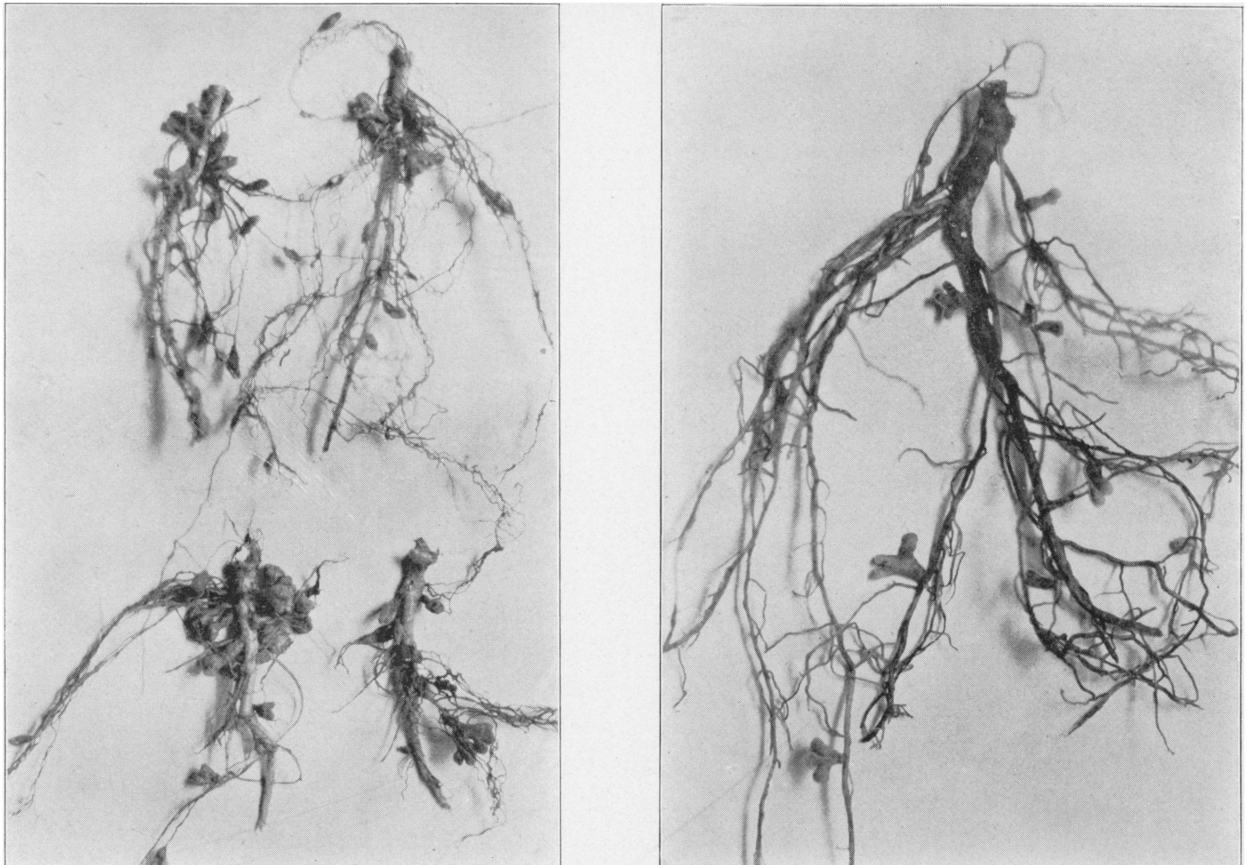
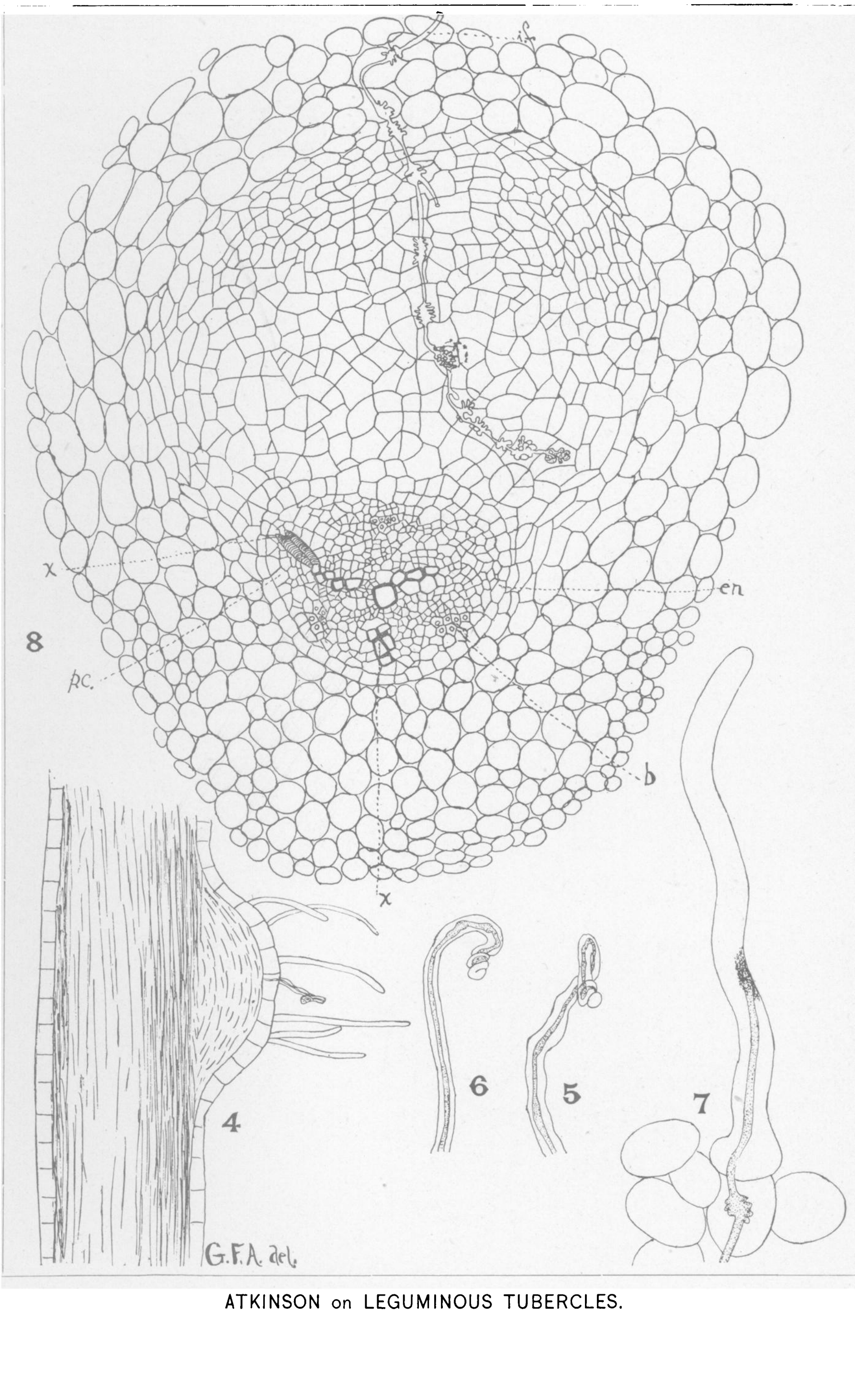


ATKINSON on LEGUMINOUS TUBERCLES.



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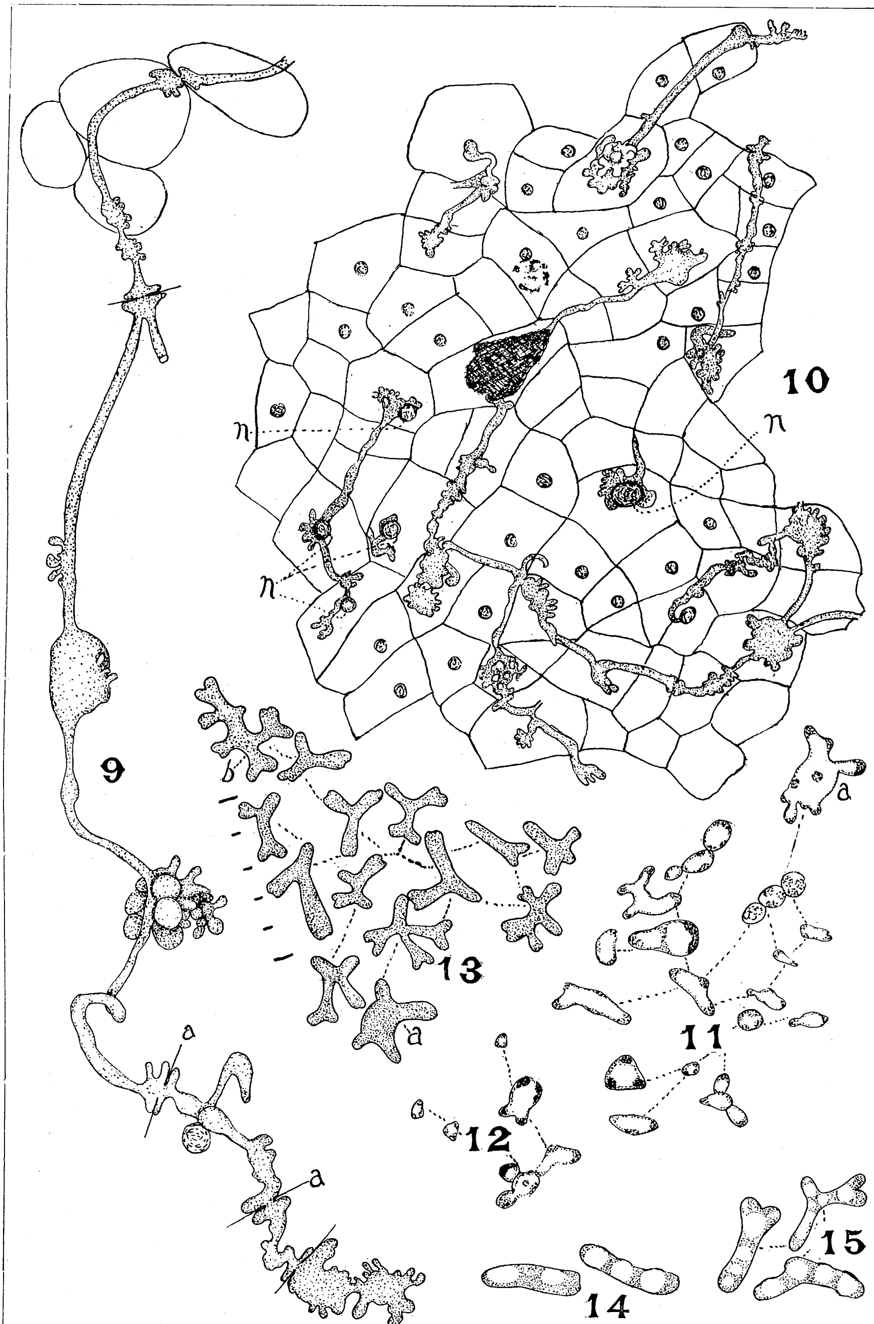

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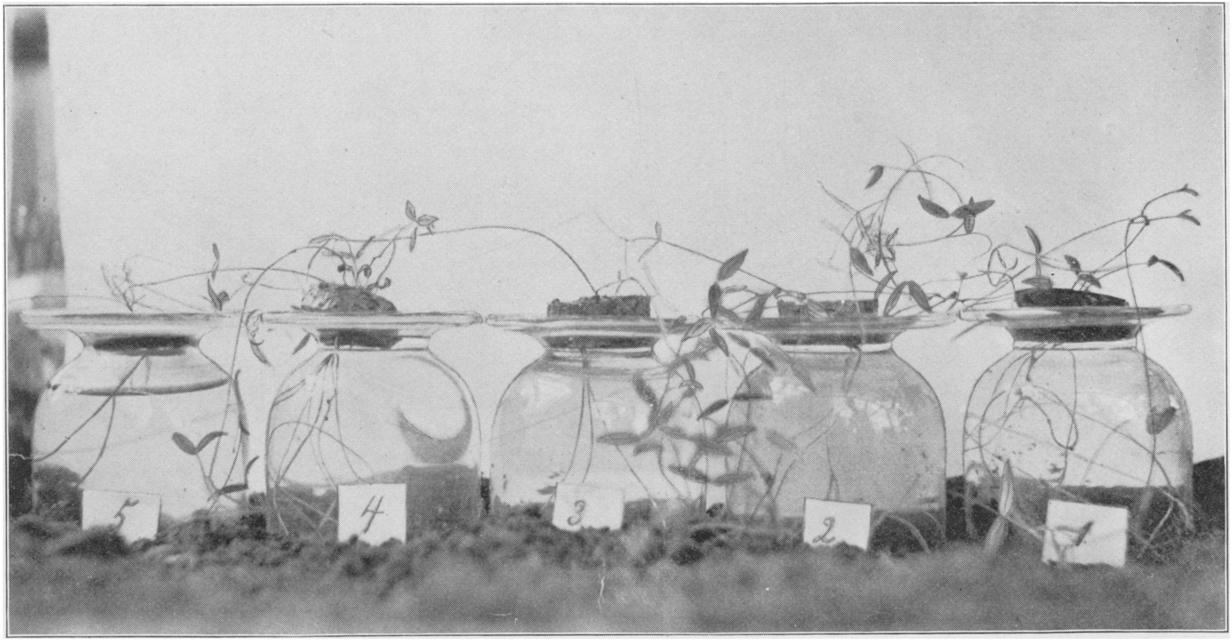

ATKINSON on LEGUMINCUS TUBERCLES.

BOTANICAL GAZETTE

MAY, 1893.

Contribution to the biology of theorganism causing leguminous tubercles.

GEO. F. ATKINSON.

WITH PLATES XII—XV.

The study of the tubercles of the Leguminosæ involves several fields of investigation. One is concerned with the economic phases of the subject, the acquisition of nitrogen from the air by plants and the enriching of the soil by decay of the plants or by the setting free of nitrogenous substances, upon the decay of the tubercles, which have been fixed by the aid of certain organisms within the tubercle. This phase of the subject is of such practical importance that it has been treated of very widely in agricultural papers and is in a general way better known than the other aspects of the subject.

Another field of investigation is a chemico-physiological one and relates to the question of the method of acquisition of nitrogen by plants, and the physiological processes involved.

Still another field of investigation is the biology of the subject. This is concerned primarily with the induction of facts relating to the morphology, development and kinship of the lowly organism, to the structural features of the tubercles and the relation of the two symbionts. It is beyond the scope of the present paper to treat of the economic and chemico-physiological aspects of the subject. The discussion will be confined mainly to the biology of the organism, since this part of the subject is the least understood and there exist so many conflicting views concerning it. The structure and development of the tubercle is better understood and will be referred to only incidentally as is found necessary in dealing with the relations of the two organisms.

Original investigations.

Four years ago while engaged upon a study of the nematode root-galls[1] of various cultivated plants, I was led to investigate with some care the morphology and anatomy of the leguminous tubercles and their contents in order primarily to discover differential characters which could easily form the basis for the accurate separation of the tubercles from nematode galls which frequently occur on the same roots. A brief résumé of the results of the leading investigations up to that time was presented along with the results of the root-gall study.

The subject became to me so fascinating at that time that during the seasons of 1891 and 1892 considerable attention was given to further personal examination of the subject, and to familiarizing myself with the literature relating to the morphology of the organism. It soon became apparent that while nearly all investigators agreed in regard to certain morphological phenomena presented in connection with the anatomy of the tubercles, several conflicting interpretations were given of them, and nearly as many views were entertained respecting the nature of the organism. For this reason, and also because no American observations had been recorded at that time, I was led to undertake some personal investigations of the biology of the organism. During the winter of 1891-2, therefore, preparations were made for this work. It has seemed best to first present here the results of my own study, and to follow this with a historical résumé and a comparison of the conclusions reached elsewhere.

The tubercles are quite prominent lateral outgrowths of the roots and present varied forms, the types of which are associated with different groups of the Leguminosæ, though the limits of these groups do not correspond in all cases with those of genera.

In *Vicia sativa*, from the tubercles of which pure cultures of the organisms were obtained, they are digitate, and either simple or forked di- or tri-chotomously. They vary in length from a size equal to their diameter to 4^{mm} to 7^{mm}. Plate XII illustrates several roots of *Vicia sativa* reproduced from photographs. It will be observed that the tubercles stand nearly at

[1]A preliminary report upon the life history and metamorphoses of a root-gall nematode, *Heterodera radicicola* (Greeff) Müll. and the injuries caused by it upon the roots of various plants. Science Contrib. Vol. I. no. I. Ala. Agr. Exp. Station, Dec. 1889.

right angles to the roots and that there is no taxic law governing their position on the roots. On some of the plants they are crowded on the root near the union with the stem. In such cases infection took place profusely at a very young stage of the seedling. On other plants very few tubercles are found near the union of the root and stem, but they are widely distributed over the root system. The roots illustrated in plate XII show this variation well.

When the tubercles are young or growing rapidly they are of a delicate flesh color, presenting a mealy aspect from numerous cells of the cortical parenchyma which are being pushed apart from their union by the rapidly growing meristematic tissue of the tubercle. When the tubercles have attained some size and age the older portion presents a light brown color in contrast with the flesh colored distal portion. Some of the other type forms of the tubercles are represented in *Medicago denticulata*, *Dolichos Sinensis*, and *Trifolium*.

In *Medicago denticulata* they are remarkable both for their size and compact botryose ramification, attaining a diameter frequently of 1^{cm} to 1.5^{cm} and bearing a striking resemblance in external appearance to the root-galls of *Alnus* which however are produced by an entirely different fungus of the genus *Frankia*, as described by Brunchorst[2], Möller[3], and the writer[4]. In *Dolichos Sinensis* the tubercles are irregularly rotund bodies. In *Trifolium procumbens*, *Carolinianum*, and other species they are cylindrical-clavate.

Vicia sativa matures its seed during the months of May and June in central Alabama[4a]. During the sunny part of dry days the mature pods by unequal contraction are suddenly split apart and the seed cast to a considerable distance. During the summer, autumn, and early winter months the seeds are gradually buried in the soil, so that during mild weather in January and February they germinate and grow so luxuriantly as to afford in some cases valuable winter grazing. The temperature of the soil at this time is sufficiently warm to permit the growth of the tubercle organism, and at an early stage

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[2] Ueber einige Wurzelanschwellungen, besonders diejenigen von Alnus und den Elæagnaceen. Unters. Bot. Inst. Tübingen. II. 151.

[3] Beitrag zur Kenntniss der *Frankia subtilis* Brunchorst. Ber.d.deutsch.bot. Gesellsch. VIII (1890). 215-224.

[4] The genus Frankia in the United States. Bulletin Torrey Bot. Club. XIX. June, 1892.

[4a] When this study was begun the writer was connected with the Ala. Polytechnic Institute, Auburn, Ala.

it is easy to find numerous rapidly growing and fresh tubercles. The temperature of the soil is sufficiently low, however, to retard the growth of many soil organisms, so that the outer surfaces of the tubercles do not contain such numbers of bacteria as are met with in the warm days of late spring. The early season then is the more favorable for the separation of the organism.

As soon as the plants had attained sufficient size to gather a small quantity of the vines an infusion was made and added to agar-agar peptone broth to afford a suitable medium in which to transplant the organism.

Method of obtaining pure cultures.

To separate the organism without contamination young but fair sized tubercles were selected. These were washed carefully and finally rinsed in distilled water and allowed to drain until the surface was not wet. A razor was then heated, not to redness, but to such a degree as would probably kill all adherent germs. A small segment was then removed from the end of the tubercle with a quick stroke of the razor so that the heat would not kill the organisms in the tubercle. As the razor was passing through the back was lifted during the stroke in order to lessen the possibility of dragging germs from the outside into the center. Then a flamed, double, sharp-pointed platinum needle was thrust into the cut end, twisted part way around, withdrawn, and with this a puncture was made in the vetch agar in culture tubes. The first transplantings were made in March, 1892, ten or twelve culture tubes being inoculated. Some of these proved to be contaminated in a few days, possessing common forms of *Bacillus*, *Micrococcus* and *Bacterium*. Others showed no evidence of growth before eight to ten days, when there appeared in a few of the culture tubes a small whitish, glistening, pearly colony at the surface of the point of inoculation. In a week more this growth had increased, having a surface extent of 2^{mm} to 3^{mm} and presented a slight convexity of surface. The growth did not extend perceptibly to more than 2^{mm} to 3^{mm} deep in the medium, but at a subsequent time spread over a greater area upon the surface. The slow growth of the organism in the artificial medium caused it to be looked on with suspicion. When the growth had attained such proportions that a small quantity could be removed without destroying the entire colony, an examination was made of the organism.

The individuals are held rather firmly together by a viscid excretion so that portions of the mass appeared "stringy" as the needle was lifted from it. They are very minute yet quite characteristic in form, and as they appeared in artificial culture are easily distinguishable from other organisms. In general appearance many of the individuals resemble some yeast, or low torula forms, yet they differ in shape and in being much smaller than any known yeast. Although so small the individuals vary greatly in size and present remarkable variations also in form. In size they vary from 0.2μ to 2μ or 3μ, and in form from a triangular outline to oval, elliptical, oblong, torulose, or variously forked and amœboid forms, as represented in figures 11 and 12 of plate XIV. A nearly homogeneous protoplasm occupies by far the larger and more central portion of the organism, while the granular protoplasm is near the periphery, but usually collected or bunched at certain points near the interior surface of the wall, these being connected by thinner sheets or threads of similar protoplasm. After some study of the different forms and observations in cell cultures it was found that these masses of granular protoplasm are almost invariably located at the growing points of the individuals, and it would be quite possible within certain limits, perhaps, from a rather simple individual to determine the more complex form it would assume at a later stage. Figures 11 and 12 represent clearly the relation of the granular protoplasm to the growing points of the individual. In figure 11*a* the individual is of an amœboid form, and the granular protoplasm bunched near the center of the figure represents in reality a downward growing process at this point, which could easily be seen by changing the focus of the microscope.

In cell cultures which were started by inoculating melted vetch agar, after a period of incubation of five to ten days there appear minute flocculose areas of growth at different points in the medium. These increase in extent until colonies of varying size occupy the medium.

The same organism has also been obtained in cell cultures of vetch agar by transplanting to them a thin section of the inner part of a tubercle, after the cortex had been removed, of course observing aseptic precautions.

In from six weeks to two months the organism was transplanted into fresh culture media to prevent death from lack of nutrition.

Inoculation experiments.

During the month of April, 1892, an experiment was put in progress to determine the relation of this organism to the orientation of the tubercles. Vetch seeds which had been gathered the previous year for this purpose were planted in two different vessels. These vessels contained sand from which the soil had been washed. The sand was saturated with distilled water to which was added a small quantity of kainite and acid phosphate, and the vessels were then steam sterilized two hours a day for three successive days. The sand was so saturated with water that it was covered with a thin layer. This was necessary in order to provide sufficient moisture for the hard dry vetch seed to germinate. After the plants had attained a length of 5^{cm} to 10^{cm}, organisms from the culture tubes of vetch nutrient agar were transplanted to vessel no. 1. An attempt was at first made with a fine needle to inoculate roots at certain points but the quantity of moisture in the sand quickly dispersed the organism over all parts of the surface of the sand. Vessel no. 2 was held as a check. There were from fifteen to twenty plants in each vessel.

During a period of a month the plants in both vessels made slow growth, being kept in a window in the laboratory, and a large bell jar covering both rested upon an uneven surface so that the air would not be kept too moist. At the end of this time a few plants in vessel no. 1 were examined and found to possess several small tubercles each. The tubercles were very much smaller than those developed in nature, as were also the vetch plants, owing probably to the artificial conditions of environment. These tubercles on the plants in vessel no. 1, developed as a result of artificial inoculation, formed the material from which a study of the organism within the tubercle was made. The examination and study of these tubercles and the organism within extended over a month's time, and it was found at the close of this period that only a few of the plants in vessel no. 1 escaped inoculation. In vessel no. 2 not a single tubercle was developed.

In July of the same season a second inoculation experiment was conducted, the vetch plants this time being grown in water culture. Five glass vessels, capacity about one pint, having a rather small neck, were fitted each with a cork which would float on the water when this rose above the neck of the vessel and partly filled the basin formed by the flaring edges.

When the water became low the cork would rest in the neck of the vessel. The corks were perforated to admit the young roots of the vetch seedlings. Mineral fertilizers consisting of acid phosphate and kainite were added to the water and the vessels were then thoroughly sterilized. Vetch seeds were germinated on moist sterilized sand, and when the seedlings were of sufficient size for the young root to reach the water, the plant was placed in the perforation of the cork. Three plants were transferred to each vessel. In a few days when it was assured that the plants were all growing nos. 1 and 4 were inoculated by transplanting with a looped platinum needle a quantity of the organisms from the pure culture to the roots of the seedlings in the perforated cork. This experiment was also successful and though the tubercles were small they were so distinct as to be readily photographed. The entire experiment was then photographed and is represented in plate XV. Two tubercles can be seen in each of jars no. 1 and 4. The checks, nos. 2, 3 and 5 possessed no tubercles. The organisms used in this second inoculation experiment were of the fifth transplanting in the culture tubes. These results would seem to afford pretty conclusive proof that the organism described above from cultures in artificial media the specific microsymbiont of the vetch tubercles.

Mode of infection.

As stated above, the material which formed the basis for the study of the organism in its natural relation to the tubercles was obtained from the inoculated plants in the first experiment, which were grown in sand culture. Roots possessing very young tubercles were mounted in water for microscopic examination of the surface. At the youngest stage examined the tubercles presented only a low convex lateral protuberance of the surface on one side as shown in figure 4 of plate XIII. The root still possessed numerous normal root hairs some of which also occupied the surface of the young tubercle. In many instances at this young stage one of the root hairs presented a very peculiar appearance. This is represented in figure 4, plate XIII, and the same root hair is drawn to a larger scale and from two different points of view in figures 5 and 6. The very tip of the hair is coiled once in a close spiral and the end has undergone further change in shape by being bent in the form of a shepherd's crook or a walking stick. The organism usually enters at the end of the root

hair, and must exert a peculiar and powerful influence in order to bring about this change in form.

Form of the microsymbiont in natural environment.

In the protoplasm of the root hair, which is a more suitable pabulum than the nutrient vetch agar, and also in point of view of economy in nature, the organism does not grow indifferently as to direction but extends straight away down the root hair in its effort to reach the cortical parenchyma of the root. Probably the macrosymbiont also exerts some influence on the direction and definiteness of the growth of the microsymbiont.

In figures 5 and 6 the organism presents the form of a thread or non-septate hypha, in size from one fourth to one third the diameter of the root hair. It describes a somewhat flexuous course and occasionally, as shown near the outer end in figure 6, broadens slightly at places presenting a nodulose appearance. Rarely the organism enters the root hair nearer the proximal end as shown in figure 7. In such cases the end of the root hair is not deformed. The ends of the root hairs frequently become deformed and variously curved from other causes, but when it is due to this organism its presence can quite easily be noted.

The contents of the infecting hypha are homogeneous and the entire thread presents a gleaming aspect which enables one to readily distinguish it from the contents of the root hair.

When the organism has once entered the cortical parenchyma its manner of growth becomes quite different, since it is coming more directly within the arena of its real activity and more directly under the influence of the macrosymbiont. By this time it has already stimulated the root tissues at that point to greater activity and a meristematic growing point arises about it within the cortical parenchyma. Frequently as the hypha passes through the cell walls it first broadens out in the form of a disk over the surface of the wall at this point, the portion of the hypha which passes through the cell wall being very slender, and upon reaching the other side of the wall it again broadens out into a disk. A lateral view presents the appearance shown in figure 8, a section of the young tubercle containing the infecting hypha. This is better shown in figure 9, the cell walls being represented in some cases by a straight transverse line. This does not occur at the passage

of all the cell walls, but it is of very common occurrence. As represented in figure 7 the hypha sometimes enlarges within the center of the cell lumen very near the epidermis. Farther within the tissue of the tubercle the enlargement of the hypha within the cell lumen is of very frequent occurrence.

The surface of these enlarged portions within the cell lumen is usually marked by a number of protuberances in the nature of buds. These buds present various forms, being either spherical, oval, pyriform, clavate, cylindrical or forked. Soon after entering the root the hypha branches. Some of these ramifications continue toward the center of the root until they reach the endodermis, but do not pass beyond. Others take a lateral course, and from these other ramifications extend outward, and as the tubercle grows in length some are directed radially from the axis of the root and grow in an opposite direction from the course pursued by the infecting thread. Thus the entire inner portion of the tubercle is traversed in a labyrinthine fashion by the ramifications of this organism. A portion of the center of the section shown in fig. 8 is more highly magnified and illustrated in detail in fig. 10, plate XIV. It is a very common thing for the hypha in passing the lumen of the cell to approach the nucleus, enlarge and partly surround it, as shown in figure 10. In many cases the nucleus seems to be stimulated so that it becomes larger than the nuclei not in contact with the hypha.

In making the section represented by figure 8, the infecting thread was cut off along with the root hair at the surface face of the tubercle and appeared in an adjacent section. This is mentioned merely to account for the absence of the root hair containing the infecting thread in that figure. These tubercles produced by inoculation in artificial culture were far superior for studying the form of the organism in the tubercle to those developed in the soil. The food and conditions for growth for the vetch being inferior to those in its natural environment, the protoplasm was not developed so profusely in the tubercle and therefore presented a more favorable opportunity for staining and definition. Violet dahlia, first suggested by Laurent[5] for the tubercle hyphæ, was used in making the preparations.

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[5]Recherches sur les nodosités radicles. Ann. de l'Inst. Pasteur, v (1891). 105–139.

While the organism does not pass the endodermis, its influence upon the hypertrophy of the tissues extends to the endodermis and the tissue of the central cylinder on that side.

(*To be continued.*)

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The genus Corallorhiza.

M. B. THOMAS.

WITH PLATES XVI AND XVII.

The genus Corallorhiza contains twelve recognized and well defined species with widely varying habitat, being found in Europe, Asia, United States and Mexico.

Four species are found in Northern United States; *Corallorhiza innata* R. Brown, *C. odontorhiza* Nutt., *C. multiflora* Nutt. and *C. striata* Lindl. Of these C. multiflora has the widest range and is the one found in greatest abundance.

The parts above ground have been quite thoroughly studied and the structure is generally well understood, but the parts below ground certainly afford an opportunity for much profitable investigation. The plants of the genus are brownish or yellowish herbs, without chlorophyll, except a little late in life, and in C. multiflora they often reach the height of eighteen inches.

The parts underground are usually described as being "much branched and toothed coral-like root-stocks (probably root parasitic) sending up a simple scape, with sheath in place of leaves, and bearing a raceme of rather small, dull-colored flowers."[1] From the rather striking characters of the stem and flowers it might be expected that the plant would exhibit several marked deviations from the regular phenomena of growth and development, and such is indeed the case. The coralline root-stock which the plant possesses has many variations from the regular type of the underground stem of monocotyledonous plants. The vascular system is represented by somewhat modified collateral bundles which are confined to the center of the stem; these are small and usually quite rudimentary. The whole vascular system is

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[1] Gray's Manual, 6th edition.